

Epratuzumab–SN-38: A New Antibody–Drug Conjugate for the Therapy of Hematologic Malignancies

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Abstract

We previously found that slowly internalizing antibodies conjugated with SN-38 could be used successfully when prepared with a linker that allows approximately 50% of the IgG-bound SN-38 to dissociate in serum every 24 hours. In this study, the efficacy of SN-38 conjugates prepared with epratuzumab (rapidly internalizing) and veltuzumab (slowly internalizing), humanized anti-CD22 and anti-CD20 IgG, respectively, was examined for the treatment of B-cell malignancies. Both antibody–drug conjugates had similar nanomolar activity against a variety of human lymphoma/leukemia cell lines, but slow release of SN-38 compromised potency discrimination *in vitro* even against an irrelevant conjugate. When SN-38 was stably linked to the anti-CD22 conjugate, its potency was reduced 40- to 55-fold. Therefore, further studies were conducted only with the less stable, slowly dissociating linker. *In vivo*, similar antitumor activity was found between CD22 and CD20 antibody–drug conjugate in mice-bearing Ramos xenografts, even though Ramos expressed 15-fold more CD20 than CD22, suggesting that the internalization of the epratuzumab–SN-38 conjugate (Emab–SN-38) enhanced its activity. Emab–SN-38 was more efficacious than a nonbinding, irrelevant IgG–SN-38 conjugate *in vivo*, eliminating a majority of well-established Ramos xenografts at nontoxic doses. *In vitro* and *in vivo* studies showed that Emab–SN-38 could be combined with unconjugated veltuzumab for a more effective treatment. Thus, Emab–SN-38 is active in lymphoma and leukemia at doses well below toxic levels and therefore represents a new promising agent with therapeutic potential alone or combined with anti-CD20 antibody therapy. *Mol Cancer Ther*; 11(1); 224–34. ©2011 AACR.

Introduction

A significant effort has focused on the biologic therapy of leukemia and lymphoma, where unconjugated antibodies (e.g., rituximab, alemtuzumab, ofatumumab), radioimmunoconjugates (⁹⁰Y-ibritumomab tiuxetan, ¹³¹I-tositumomab), and a drug conjugate (gemtuzumab ozogamicin) received U.S. Food and Drug Administration (FDA) approval. Another antibody–drug conjugate (ADC), brentuximab vedotin (SGN-35; anti-CD30-auristatin E; refs. 1, 2), recently received accelerated approval by the FDA for Hodgkin lymphoma and anaplastic large-cell lymphomas. There are also a number of other ADCs in preclinical and clinical development that target CD19, CD22, CD37, CD74, and CD79b (3, 4).

Antibodies against all of these targets are logical choices for carriers of drugs, because they are internalizing (5). Internalization and specificity of CD22 have made it a particularly important target for leukemia and lymphomas, with at least 3 different anti-CD22 conjugates in clinical investigation, including CMC-544 (acid-labile-conjugated calicheamicin), an anti-CD22-maytansine conjugate (stably linked MCC-DM1), and CAT-3888 (formally BL22; a *Pseudomonas* exotoxin single-chain fusion protein; refs. 6–8). The active agent in all of these conjugates has subnanomolar potency (i.e., so called ultra-toxics).

We recently developed methods to conjugate antibodies with SN-38, a topoisomerase I inhibitor with low nanomolar potency that is derived from the prodrug, irinotecan (9, 10). Four SN-38 linkage chemistries were examined initially using conjugates prepared with a slowly internalizing anti-CEACAM5 antibody (9, 10). The conjugates retained CEACAM5 binding but differed in the dissociation rate of SN-38 in human serum, with half-lives varying from approximately 10 to 67 hours (9). Ultimately, the linker designated CL2, with intermediate stability (~50% dissociated in 24–35 hours), was selected for further development. CL2 was modified recently, eliminating the phenylalanine in the cathepsin B–cleavable dipeptide to simplify and improve manufacturing yields (11). The new derivative, designated CL2A, retains the pH-sensitive carbonate linkage to the SN-38, but it is

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no longer selectively cleaved by cathepsin B. Nevertheless, it has identical serum stability and *in vivo* activity as the original CL2 linker (11). Because significant efficacy without toxicity was found with the slowly internalizing anti-CEACAM5–SN-38, we postulated that its activity was aided by the slow release of SN-38 from the antibody after it localized in a tumor (9, 11). Thus, the main objective in this report was to evaluate the therapeutic prospects of conjugates prepared using the CL2A linker with two antibodies that are highly specific for B-cell cancers but differ in their antigen expression and internalization properties.

Epratuzumab (Emab) is a rapidly internalizing (e.g., $\geq 50\%$ within 1 hour), humanized anti-CD22 IgG₁ that has been evaluated extensively in lymphoma and leukemia in an unconjugated or conjugated form (12–22). Veltuzumab (Vmab) is a humanized anti-CD20 antibody that is also being studied clinically (23, 24) but internalizes slowly (e.g., $\sim 10\%$ in 1 hour; refs. 15, 25). CD20 is usually expressed at much higher levels than CD22 in non-Hodgkin lymphoma (NHL; refs. 26–28), whereas CD22 is preferentially expressed in acute lymphoblastic leukemia (ALL) but not in multiple myeloma (29, 30). Both antibodies are effective in patients as unconjugated agents, but only veltuzumab is active in murine xenograft models (31). On the basis of previous studies that showed ⁹⁰Y-Emab combined with unconjugated veltuzumab had enhanced efficacy in NHL models (16, 17), we also examined the Emab–SN-38 + Vmab combination, as this could provide additional benefit without competing for the same target antigen or having additional toxicity.

Materials and Methods

Cell lines

Ramos, Raji, Daudi (Burkitt lymphomas), and JeKo-1 (mantle cell lymphoma) were purchased from American Type Culture Collection. REH, RS4;11, MN-60, and 697 (ALL) were purchased from Deutsche Sammlung von Mikroorganismen und Zellkulturen. WSU-FSCCL (follicular NHL) was the gift of Dr. Mitchell R. Smith (Fox Chase Cancer Center, Philadelphia, PA). All cell lines were cultured in a humidified CO₂ incubator (5%) at 37°C in recommended supplemented media containing 10 to 20% fetal calf serum and were checked periodically for Mycoplasma.

Antibodies and conjugation methods

Epratuzumab and veltuzumab are humanized anti-CD22 and anti-CD20 IgG₁ monoclonal antibodies, respectively (13, 25, 31–33). Labetuzumab (Lmab), a humanized anti-CEACAM5 IgG₁, and RS7, a humanized anti-Trop-2 antibody (both from Immunomedics, Inc.), were used as nonbinding, irrelevant controls (9, 11). Herein, Emab–SN-38, Vmab–SN-38, and Lmab–SN-38 refer to conjugates prepared using the CL2A linker that was described recently (11). *In vitro* studies in human serum showed that approximately 50% of the active SN-38 moiety is

released from the IgG each day (11). Another linker, designated CL2E, is stable in human serum over 14 days (34), but it contains a cathepsin B cleavage site to facilitate the release of SN-38 when processed in lysosomes. The method to prepare CL2E and the structures of the CL2A and CL2E linkers are given in Supplementary Methods and Supplementary Figs. S1 and S2. The conjugates contain approximately 6 SN-38 units per IgG (e.g., 1.0 mg of the IgG–SN-38 conjugate contains $\sim 16 \mu\text{g}$ of SN-38).

In vitro cell binding and cytotoxicity

Flow cytometry was carried out using the unconjugated specific and irrelevant antibodies incubated for 1 hour at 4°C, with binding revealed using fluorescein isothiocyanate (FITC)-Fc γ fragment-specific goat anti-human IgG (Jackson ImmunoResearch), also incubated for 1 hour at 4°C. Median fluorescence was determined on a FACSCalibur flow cytometer (Becton Dickinson) using CellQuest software package.

Cytotoxicity was determined using the MTS dye reduction assay (Promega), as described previously (11). Dose-response curves [with/without goat anti-human Fc γ F(ab')₂; Jackson ImmunoResearch] were generated from the mean of triplicate determinations, and IC₅₀-values were calculated using Prism GraphPad software (v5), with statistical comparisons using an *F* test on the best fit curves for the data. Significance was set at *P* < 0.05.

Immunoblotting

After 24- or 48-hour exposure to the test agents, markers of early (p21 expression) and late (PARP cleavage) apoptosis were revealed by Western blotting, as described previously (11).

In vivo studies

All studies were conducted in accordance with protocols approved by the institutional animal care and use committee of Center for Molecular Medicine and Immunology. The subcutaneous Ramos model was initiated by implanting 1×10^7 cells (0.2 mL) from culture (>95% viability) into 4- to 6-week-old female nude mice (Taconic). Three weeks from implantation, animals with tumors ranging from 0.4 to 0.8 cm³ (measured by caliper, $L \times W \times D$) were segregated into groups of animals, each with the same range of tumor sizes. Tumor size and body weights were measured at least once weekly, with animals removed from the study when tumors grew to 3.0 cm³ or if they experienced 20% or greater body weight loss. The intravenous WSU-FSCCL and 697 models were initiated by intravenous injection of 2.5×10^6 and 1×10^7 cells, respectively, in female severe combined immunodeficient (SCID) mice (Taconic). Treatment began 5 days after administration of the WSU-FSCCL cells and 7 days after the 697 inoculation. Animals were observed daily, using hind leg paralysis or other signs of morbidity as surrogate survival endpoints. All treatments were given intraperitoneally in ≤ 0.2 mL. The specific dosages and frequency are given in the Results section. Because mice

convert irinotecan to SN-38 efficiently (35, 36), irinotecan dosing was adjusted on the basis of SN-38 equivalents; SN-38 mole equivalents are based on 1.6% of ADC mass and 60% of irinotecan mass.

Efficacy was expressed in a Kaplan–Meier curve, using time to progression (TTP) as surrogate survival endpoints as indicated above. Statistical analysis was conducted by a log-rank test using Prism GraphPad software (significance, $P < 0.05$).

Results

Antigen expression and cytotoxicity *in vitro*

All cell lines were highly susceptible to SN-38, with EC_{50} values ranging from 0.13 nmol/L for Daudi to 2.28 nmol/L for RS4;11 (Table 1). Except for 697 and RS4;11, the Emab–SN-38 anti-CD22 conjugate was 2- to 7-fold less effective than SN-38. This is a common finding with our targeted, as well as other nontargeted, SN-38 conjugates (11, 37–39). Despite differences in antigen expression, the Emab–SN-38 and Vmab–SN-38 had similar potencies as the nonbinding, Lmab–SN-38 anti-CEACAM5 conjugate, which was likely due to dissociation of approximately 90% of SN-38 during the 4-day MTS assay. Other *in vitro* procedures using shorter exposure times were also ineffective in discriminating differences in the potencies of conjugates. For example, Annexin V staining after a 1-day exposure failed to find differences between untreated and treated cells (not shown). Upregulation of p21 and PARP cleavage was also examined as early and late markers of apoptosis, respectively. Ramos did not express p21. However, PARP cleavage was detected, but only after a 48-hour exposure, being more strongly expressed in SN-38–treated cells (Supplementary Fig. S3). The WSU-FSCCL cell line expressed p21, but neither p21 upregulation nor PARP cleavage was evident until 48 hours after Emab–SN-38 exposure. However, both were observed after a 24-hour exposure with free SN-38 (Supplementary Fig. S4B). While the enhanced intensity and earlier activation of apoptotic events with free SN-38 are consistent with its lower EC_{50} over the IgG-conjugated form, the results indicated that an exposure period of at least 48 hours would be required, but at this time, approximately 75% of the SN-38 would be released from the conjugate.

To assess the prospect for enhanced cytotoxicity when Emab–SN-38 is combined with unconjugated anti-CD20 antibody, cells were co-incubated with velvuzumab and increasing concentrations of Emab–SN-38. A cross-linking anti-human IgG antibody was added to the reaction mixture to simulate *in vivo* binding events that trigger apoptosis with unconjugated antibodies (32, 40, 41). Mortality with cross-linked velvuzumab alone ranged from 20% to 35% of the untreated cells, plateauing in its anti-proliferative effect at 1 to 10 nmol/L (WSU-FSCCL was unaffected at 200 nmol/L, the highest concentration tested). Cross-linked, unconjugated epratuzumab had no effect on cell viability, but Emab–SN-38 killed 100% of the cells at approximately 1 to 10 nmol/L. With the exception

of WSU-FSCCL, the IC_{50} improved with the Emab–SN-38 + Vmab combination (Table 2). Daudi was affected the most (~10-fold), with approximately 2-fold increase in mortality in the other 3 cell lines. Only Raji responded to higher amounts of velvuzumab.

We again examined PARP cleavage and p21 expression, this time in cells treated with Emab–SN-38 + Vmab. Confirming the earlier study (Supplementary Fig. S3) in Ramos, PARP cleavage first occurs only after a 48-hour exposure to the conjugate, with expression unchanged in the presence of a cross-linking antibody (Supplementary Fig. S4A). Exposure to velvuzumab for more than 48 hours had no effect on PARP cleavage (lanes 8 and 9), but cleavage was strong within 24 hours when a cross-linking antibody was added (lane 10). However, when velvuzumab alone (no cross-linker) was combined with Emab–SN-38, PARP cleavage occurred after a 24-hour exposure (lane 12), indicating velvuzumab could induce a more rapid onset of apoptosis, even in the absence of cross-linking. The only notable difference in the WSU-FSCCL cell line was that the combination greatly enhanced p21 expression at 48 hours (Supplementary Fig. S4B), again suggesting an acceleration of apoptosis induction when velvuzumab is combined with the Emab–SN-38 conjugate. The delay in apoptosis induction in WSU-FSCCL as compared with Ramos is likely explained by the lower expression of CD22 and CD20.

Ultratotoxic agents often use linkers that are highly stable in serum, as their premature release would increase toxicity, but these conjugates must be internalized for the drug to be delivered optimally (5). Because epratuzumab internalizes rapidly, we examined whether it might benefit from a more stably linked SN-38, comparing *in vitro* cytotoxicity of the CL2A-linked Emab–SN-38 conjugate with the serum-stable CL2E–SN-38 conjugate. Both conjugates had a similar binding affinity (Supplementary Data), but the more stable Emab–CL2E–SN-38 was approximately 40- to 55-times less potent than the CL2A conjugate in 3 cell lines. While specificity was lacking with the CL2A conjugates, the Emab–CL2E–SN-38 consistently was approximately two times more potent than the non-binding Lmab–anti-CEACAM5–CL2E–SN-38 conjugate (Table 3). We concluded that it was unlikely that the more stably linked conjugate would be appropriate for a slowly internalizing velvuzumab conjugate and therefore continued our investigation only with CL2A-linked SN-38 conjugates.

Emab anti-CD22–SN-38 versus Vmab anti-CD20–SN-38 in subcutaneous Ramos xenografts

Because of limitations of the *in vitro* assays, efficacy was assessed in xenograft models. As indicated in Table 1, all of the lymphoma cell lines have much higher expression of CD20 than CD22. Daudi had the highest expression of CD22 and CD20, but it is very sensitive *in vivo* to unconjugated velvuzumab (32) and *in vitro* testing revealed the highest sensitivity to SN-38 (Table 1). These properties would likely make it difficult to assess differences in

Table 1. Expression of CD20 and CD22 by FACScan and *in vitro* cytotoxicity by MTS assay of SN-38 and specific Emab anti-CD22-SN-38, Vmab anti-CD20-SN-38, and Lmab anti-CEACAM5-SN-38 conjugates against several hematopoietic tumor cell lines

Cell line	CD20 expression		CD22 expression		EC ₅₀ values ^a					
	Median fluorescence (background)	SN-38, nmol/L	Median fluorescence (background)	SN-38, nmol/L	Emab-SN-38, nmol/L	95% CI	Vmab-SN-38, nmol/L	95% CI	Lmab-SN-38, nmol/L	95% CI
NHL:Burkitt										
Raji	422.2 (6.8)	1.42	45.9 (6.8)	1.42	2.10	0.8-2.4	ND	1.2-3.8	ND	—
Ramos	620.4 (4.1)	0.40	40.8 (4.1)	0.40	4.61	0.2-0.7	4.88	2.2-9.5	3.73	1.8-7.6
Daudi	815.1 (5.9)	0.13	145.0 (5.9)	0.13	2.92	0.1-0.2	ND	1.6-5.4	ND	—
NHL:follicular					9.84	0.1-0.2	13.56	4.5-21.6	8.08	2.9-22.2
WSU-FSCCL	97.4 (4.9)	0.50	7.7 (4.9)	0.50	0.52	0.3-1.0	ND	0.4-0.7	ND	—
NHL:mantle cell					1.05	0.3-1.0	0.83	0.4-1.1	ND	—
Jeko-1	604.6 (6.5)	ND	11.2 (6.5)	ND	2.25	—	1.98	0.8-1.4	1.17	0.8-1.7
ALL:B cell								1.3-3.8	2.27	1.3-3.9
REH	12.3 (4.1)	0.47	22.9 (4.1)	0.47	1.22	0.3-0.9	ND	0.8-1.9	ND	—
697	6.9 (4.2)	2.23	16.0 (4.2)	2.23	2.67	1.3-3.9	ND	1.7-3.7	ND	—
RS4;11	3.7 (4.1)	2.28	23.3 (4.1)	2.28	1.68	1.1-4.9	ND	1.0-3.0	ND	—
MN-60	21.5 (5.8)	1.23	10.3 (5.8)	1.23	3.65	0.6-2.1	ND	2.2-6.2	ND	—

Abbreviations: CI, confidence interval; ND, not determined.

^aEC₅₀ expressed as mole equivalents of SN-38 in Emab-SN-38.

Table 2. Potency of Emab–SN-38, anti-CD22–SN-38 conjugate co-incubated with unconjugated Vmab anti-CD20 IgG

Treatment ^b	IC ₅₀ values ^a , nmol/L				
	Raji	Ramos	Daudi	WSU-FSCCL	JeKo-1
Emab–SN-38	1.72	1.84	0.50	0.38	1.77
Emab–SN-38 + Vmab (133 nmol/L) ^c	1.16	0.73	0.06	0.41	0.81
Emab–SN-38 + Vmab (1.33 μmol/L) ^c	0.19	0.51	0.20	0.34	0.83
Emab–SN-38 + hRS7 (133 nmol/L)	2.88	2.21	0.84	0.46	1.66
Emab–SN-38 + hRS7 (1.33 μmol/L)	2.45	2.29	1.50	0.46	1.16

^aIC₅₀ value is expressed as mole equivalents of SN-38 in Emab–SN-38.

^bGoat anti-human IgG was added to each treatment. Humanized RS7 (hRS7) anti-Trop-2 is a nonbinding human IgG₁.

^cMortality of cells treated with only cross-linked veltuzumab was examined at concentrations up to 200 nmol/L, with maximum mortality (<35%) plateauing between 1 and 10 nmol/L. Cross-linked epratuzumab alone had no effect on mortality. The combination of epratuzumab with veltuzumab and a cross-linker was similar to veltuzumab alone (not shown).

activity attributed to the SN-38 conjugate versus the unconjugated antibody, particularly when unconjugated epratuzumab is not an effective therapeutic in animals (31). Because Ramos had been used previously to show an advantage for combining ⁹⁰Y-Emab with veltuzumab (16), we elected to start with a comparison of the Emab–SN-38 and Vmab–SN-38 conjugates in the Ramos human Burkitt cell line. Despite flow cytometry showing a 15-fold higher expression of CD20 over CD22, immunohistology of Ramos xenografts showed abundant CD22 and CD20, with CD22 seemingly expressed more uniformly than CD20 (Supplementary Fig. S5).

Ramos xenografts in untreated animals progressed rapidly, reaching the 3.0-cm³ termination size from their starting size of 0.4 cm³ within 6 days (not shown), and as reported previously, neither veltuzumab nor epratuzumab appreciably affected the progression of well-established Ramos xenografts (42). Consistent with previous findings using other SN-38 conjugates (9, 11), none of the

animals treated with a 4-week, twice-weekly, 0.5 mg/dose treatment regimen had appreciable weight loss. Both conjugates were highly effective in controlling tumor growth, with 80% or more of the animals having no evidence of tumor by the end of the 4-week treatment (Fig. 1). The 0.25-mg Vmab–SN-38 dose was better at controlling growth over the first 4 weeks, but at 0.5 mg, similar early growth control was observed for both conjugates. Thus, despite a 15-fold higher expression of CD20 than CD22, Emab–SN-38 compared favorably with Vmab–SN-38. Therefore, the remaining studies focused on Emab–SN-38 alone or in combination with unconjugated veltuzumab.

Emab–SN-38 dose-response and specificity

A dose-response relationship was seen for the specific Emab–SN-38 and irrelevant Lmab–SN-38 conjugates, but Emab–SN-38 had significantly better growth control at 2 of the 3 levels tested, and with a strong trend favoring the

Table 3. The effect of linkage stability on the cytotoxicity of antibody conjugates as determined by a 4-day MTS assay

Agent ^a	Ramos		Raji		REH	
	EC ₅₀ ^b	95% CI	EC ₅₀	95% CI	EC ₅₀	95% CI
SN-38	9.1	6.0–13.9	1.6	1.0–2.5	0.6	0.3–1.1
Emab–CL2A–SN-38	2.7	1.7–4.2	3.2	2.0–5.0	1.5	0.9–2.5
Emab–CL2E–SN-38	152.3	88–280	135.8	87–211	77.9	83–114
Lmab–CL2A–SN-38	5.1	3.4–7.8	2.8	1.7–4.6	1.7	0.9–3.0
Lmab–CL2E–SN-38	271.0	122–600	246.3	119–509	162.0	89–294

Abbreviation: CI, confidence interval.

^aEmab, epratuzumab anti-CD22 (binding/rapidly internalizing); Lmab, anti-CEACAM5 (nonbinding); CL2A linker allows for slow dissociation of SN-38 from the conjugate, whereas CL2E linker is very stable in serum.

^bEC₅₀ expressed as nanomolar SN-38 equivalents.

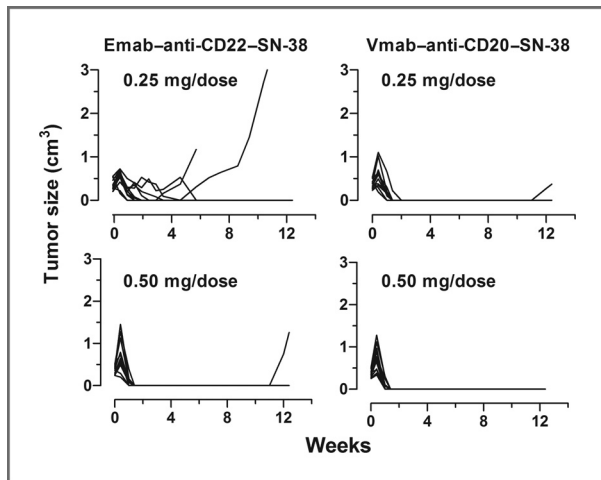


Figure 1. Comparative efficacy of Emab-SN-38 and Vmab-SN-38 conjugates in the subcutaneous Ramos model. Nude mice ($n = 10$ per group) with tumors averaging approximately 0.35 cm^3 ($0.20\text{--}0.55 \text{ cm}^3$) were administered 0.25 or 0.5 mg of each conjugate twice weekly for 4 weeks.

specific conjugate at the intermediate dose (Fig. 2). Again, 0.25 mg of Emab-SN-38 ablated a majority of the tumors; here, 7 of 10 animals were tumor-free at the end of the 12-week monitoring period, with no change in body weight. Animals given irinotecan alone ($6.5 \mu\text{g}/\text{dose}$; approximately the same SN-38 equivalents as 0.25 mg of conjugate) had a median survival of 1.9 weeks, with 3 of 11 animals tumor-free at the end of the study, which was not significantly different from the 3.45-week median survival for the irrelevant Lmab-SN-38 conjugate ($P = 0.452$; Fig. 2C).

In the 697-disseminated leukemia model, the median survival of saline-treated animals was just 17 days from tumor inoculation. Animals given unconjugated epratuzumab plus irinotecan (same mole equivalents of SN-38 as 0.5 mg of the conjugate) had the same median survival, whereas animals given 0.5 mg of Emab-SN-38 twice weekly starting 7 days from tumor inoculation survived to 24.5 days, significantly longer than untreated animals ($P < 0.0001$) or for unconjugated epratuzumab given with irinotecan ($P = 0.016$). However, Emab-SN-38 was not significantly better than the irrelevant conjugate (median survival = 22 days; $P = 0.304$), most likely reflecting the low expression of CD22 in this cell line.

Emab-SN-38 combined with unconjugated Vmab anti-CD20

We previously reported improved responses when ^{90}Y -Emab was combined with unconjugated veltuzumab in the subcutaneous Ramos model (16) and thus this possibility was examined with Emab-SN-38. In a pilot study, 5 animals bearing subcutaneous Ramos tumors averaging approximately 0.3 cm^3 were given veltuzumab (0.1 mg), 0.1 mg of Emab-SN-38, or Emab-SN-38 + Vmab (all agents given twice weekly for 4 weeks). The median TTP

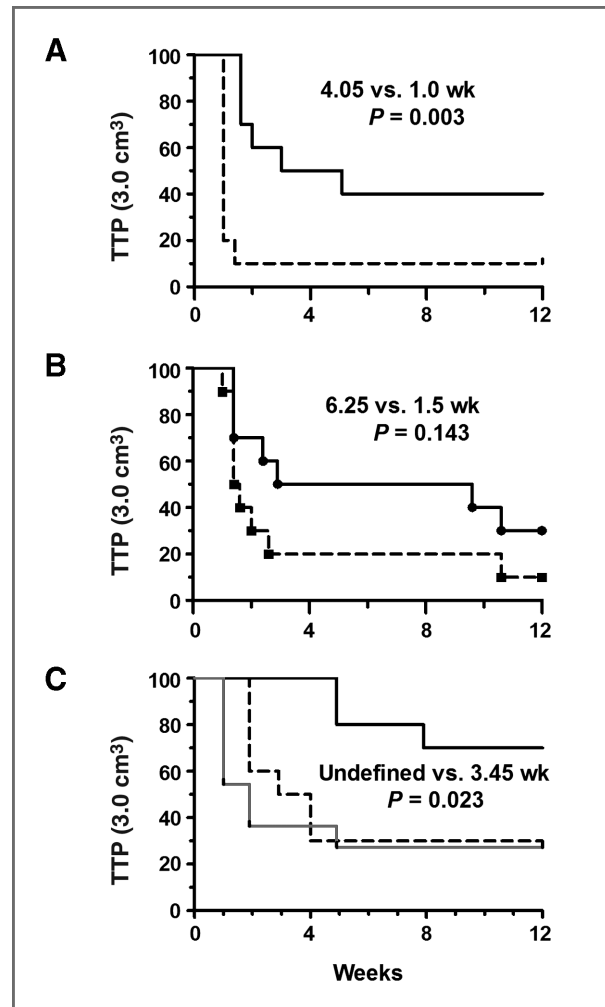


Figure 2. Specificity of Emab anti-CD22-SN-38 conjugate (solid line) versus an irrelevant Lmab-SN-38 conjugate (dashed line) in nude mice bearing subcutaneous Ramos tumors. Animals were given twice weekly doses of the conjugates intraperitoneally for 4 weeks. A, B, and C were given 75, 125, and 250 μg of each conjugate per dose (54.5, 91, and 182 $\mu\text{g}/\text{kg}$ of SN-38, respectively, based on average weight of 22 g). Survival based on TTP to 3.0 cm^3 , with tumors starting at an average size of 0.4 cm^3 . P values comparing median survival (shown) for Emab-SN-38 to Lmab-SN-38 conjugate are shown in each panel. C, survival curves (solid gray) for another group of animals given weekly intraperitoneal injections of irinotecan ($6.5 \mu\text{g}/\text{dose}$; SN-38 equivalents approximately the same as the 250- μg dose of the Emab-SN-38 conjugate).

to 2.0 cm^3 was 22, 14, and more than 77 days, respectively (veltuzumab vs. Emab-SN-38 alone, $P = 0.59$; Emab-SN-38 + Vmab vs. Emab-SN-38, $P = 0.0145$), providing an initial indication that the combination of veltuzumab with Emab-SN-38 improved the overall therapeutic response. In a follow-up study that also used a twice-weekly, 4-week treatment regimen, 6 of 11 animals given 0.1 mg of Emab-SN-38 plus 0.1 mg of veltuzumab had no evidence of tumors 16 weeks from the start of treatment, whereas the median survival for animals receiving veltuzumab alone or with 0.1 mg of the control Lmab-SN-38 was 1.9 and 3.3 weeks, respectively, with 3 of 11 animals being tumor-free

Table 4. Survival assessment in severe combined immunodeficient mice implanted intravenously with the follicular lymphoma cell line WSU-FSCCL and treated 5 days later with the agents listed

	Median survival, d		No. of survivors/total	
	Exp 1	Exp 2	Exp 1	Exp 2
Untreated	42	40	0/10	0/15
Irinotecan alone	ND	49	ND	1/15
Veltuzumab alone	91	77	2/10	0/15
Epratuzumab + irinotecan + veltuzumab	ND	77	ND	0/15
Lmab-SN-38 alone - A	49	ND	0/10	ND
Lmab-SN-38 alone - B	63	49	0/10	0/15
Lmab-SN-38 A + Vmab	98	ND	2/10	ND
Lmab-SN-38 B + Vmab	91	91	2/10	1/15
Emab-SN-38 alone - A	42	ND	0/10	ND
Emab-SN-38 alone - B	63	75	0/10	0/15
Emab-SN-38 A + Vmab	140	ND	3/10	ND
Emab-SN-38 B + Vmab	>161	126	6/10	2/15

NOTE: All treatments given intraperitoneally twice weekly for 4 weeks; combinations are given on the same day. Veltuzumab, 35 μ g/dose; unconjugated epratuzumab, 0.3 mg/dose. Lmab-SN-38 and specific Emab-SN-38 conjugates: A, 0.15 mg/dose (2.4 μ g SN-38 equivalents); B, 0.30 mg/dose (4.8 μ g SN-38 equivalents). Irinotecan, 8 μ g/dose (5.3 μ g SN-38 equivalents). Abbreviation: ND, not determined.

at 16 weeks in each of these groups (Supplementary Fig. S6). Despite the longer median TTP and more survivors, no significant differences were found between the groups. Thus, in the Ramos model, which has abundant CD20 and moderate levels of CD22, the Emab-SN-38 conjugate given at nontoxic dose levels was not significantly better than unconjugated anti-CD20 therapy, but the addition of Emab-SN-38 to unconjugated anti-CD20 therapy appeared to improve the response without toxicity. It is important to emphasize that the SN-38 conjugates are given at levels far less than their maximum tolerated dose (11), and therefore these results should not be interpreted that the unconjugated anti-CD20 therapy is equal to that of the Emab-SN-38 conjugate.

Two additional studies were conducted in an intravenous implanted model using the WSU-FSCCL follicular NHL cell line that has a low expression of CD20 and CD22 (Table 4; Supplementary Fig. S7). The median survival time for saline-treated animals was 40 to 42 days from tumor implantation. Irinotecan alone (Table 4, Exp 2), given at a dose containing the same SN-38 equivalents as 0.3 mg of the ADC, increased the median survival (49 vs. 40 days, respectively; $P = 0.042$), but 14 of 15 animals succumbed to disease progression on day 49, the same day the final 4 of 15 animals in the saline group were eliminated (Supplementary Fig. S7). Despite its relatively low CD20 expression, veltuzumab alone (35 μ g twice weekly \times 4 weeks) was effective in this model. The median survival increased to 91 days in the first study, with 2 cures (day 161), and to 77 days in the second, but with no survivors after 89 days (veltuzumab alone vs. saline-treated, $P < 0.001$ in both studies). Unconjugated

epratuzumab (0.3 mg/dose) combined with irinotecan and veltuzumab had the same median survival as veltuzumab alone, suggesting that neither epratuzumab nor irinotecan contributed to the net response.

As expected because of the low CD22 expression by WSU-FSCCL, Emab-SN-38 alone was not as effective as in Ramos. At the 0.15-mg dose, no significant benefit over the saline group was seen, but at 0.3 mg, the median survival increased to 63 days, providing a significant improvement compared with the saline-treated animals (Table 4, Exp 1; $P = 0.006$). The second study, using 0.3 mg of Emab-SN-38, confirmed an enhanced survival compared with the saline group (75 vs. 40 days; $P < 0.0001$). The specificity of this response was not apparent in the first study, where the median survival of the irrelevant Lmab-SN-38 conjugate and Emab-SN-38 were not different at either 0.15- or 0.3-mg dose levels (42 vs. 49 days and 63 vs. 63 days for the Emab-SN-38 vs. anti-CEACAM5-SN-38 conjugates at the 2 doses levels, respectively). However, in the second study, the 0.3-mg dose of Emab-SN-38 provided a significantly improved survival over the irrelevant conjugate (75 vs. 49 days; $P < 0.0001$). Again, the difficulty in showing specificity in this model is most likely related to low CD22 expression.

Combining the specific Emab-SN-38 with veltuzumab substantially increases survival, with evidence of more robust responses than the control Lmab-SN-38. For example, in the first study, animals treated with veltuzumab plus 0.15 or 0.3 mg of the control conjugate had a median survival of 98 and 91 days, respectively, which was similar to that of veltuzumab alone (91 days; Table 4, Exp 1). However, veltuzumab plus 0.15

mg of the specific Emab–SN-38 conjugate increased the median survival to 140 days. While this improvement was not significantly higher than veltuzumab alone ($P = 0.257$), when the Emab–SN-38 dose was increased to 0.3 mg with veltuzumab, 6 of 10 animals remained alive at the end of the study, providing a significant survival advantage over the control conjugate plus veltuzumab ($P = 0.0002$). In a second study, the median survival of veltuzumab alone was shorter than in the first (77 vs. 91 days), yet the median survival for the control conjugate with veltuzumab was again 91 days, which now yielded a significant survival advantage over veltuzumab alone ($P < 0.0001$; Table 4, Exp 2). Combining the specific Emab–SN-38 conjugate with veltuzumab extended the median survival to 126 days, which was significantly longer than the median survival of 75 and 77 days for Emab–SN-38 and veltuzumab alone, respectively ($P < 0.0001$ for each). However, in this study, it did not quite meet the requirements for a statistical improvement over the combination with control anti-CEACAM5–SN-38 conjugate ($P = 0.078$).

Discussion

Over the past 10 years, ADCs have made substantial gains in cancer therapy, yet there also have been some setbacks. The gains occurred largely when investigators chose to examine agents that were too toxic to be used alone, but when coupled to an antibody, these so-called ultratoxics produced substantially improved responses in preclinical testing. The recent approval of brentuximab vedotin, an auristatin conjugate, in Hodgkin lymphoma and the clinical success with trastuzumab–DM1 anti-HER2–maytansine conjugate as a single agent in breast cancer refractive to unconjugated trastuzumab suggest that these ADCs bearing ultratoxic agents are becoming accepted treatment modalities (43). However, conjugates prepared with agents that are themselves potent in the picomolar range can have an increased risk for toxicity, as the recent decision to withdraw gemtuzumab ozogamicin, the anti-CD33–calicheamicin conjugate, from the market suggests (44). Thus, the success of an ADC will depend on identifying appropriate chemistries to bind the drug and antibody together, as well as defining a suitable target that is sufficiently expressed to allow an adequate and selective delivery of the cytotoxic agent.

We developed a linker for coupling SN-38 to IgG that allows SN-38 to be released slowly from the conjugate in serum (~50% per day). With this linker, an antibody that is slowly internalized could be an effective therapeutic (9, 10), perhaps because the conjugate localized to a tumor releases a sufficient amount of drug locally, even without being internalized. The CL2A linker also was used recently with an antibody to Trop-2 that was reported to be internalized rapidly (11, 45). Thus, it appears that the slow release mechanism is beneficial for internalizing and noninternalizing antibodies.

In this report, we expanded our assessment of the CL2A linker by comparing SN-38 conjugates prepared with

epratuzumab, a rapidly internalizing anti-CD22 IgG (22), and veltuzumab, a slowly internalizing anti-CD20 IgG (32), for the treatment of B-cell malignancies. Prior studies with the murine parent of epratuzumab had indicated that most of the antibody internalizes within 1 hour and 50% of CD22 is reexpressed on the cell surface within 5 hours (22). This internalization and reexpression process would permit intracellular delivery that might compensate for lower surface expression of CD22. Because many of the B-cell malignancies express much more CD20 than CD22, a conjugate targeting CD20 might deliver more moles of drug by releasing its toxic payload after being localized in the tumor.

In vitro cytotoxicity studies could not discriminate the potency of the specific conjugates or even an irrelevant conjugate because of the release of SN-38 from the conjugate into the media. Indeed, SN-38 alone was somewhat more potent than the conjugates, which may reflect its accelerated ability to enter the cell and engage topoisomerase I. Because other studies revealed that the conjugates required a 48-hour exposure before early signs of apoptosis could be seen, we concluded that *in vitro* testing would not be able to discriminate the potency of these 2 conjugates and therefore resorted to *in vivo* studies.

In xenograft models, both conjugates had similar antitumor activity against Ramos tumors, which flow cytometry had indicated expressed nearly 15-fold more CD20 than CD22. This lent support to selecting the Emab anti-CD22–SN-38 conjugate especially because it could be combined with unconjugated Vmab anti-CD20 therapy without concern that either agent would interfere with the binding of the other agent. Indeed, if an anti-CD20–SN-38 conjugate were used, the total IgG protein dose given likely would be below a level typically needed for effective unconjugated anti-CD20 antibody treatments, as the dose-limiting toxicity would be driven by the SN-38 content. Adding more unlabeled anti-CD20 to an anti-CD20–SN-38 conjugate would risk reducing the conjugate's uptake and potentially diminishing its efficacy. However, as we showed previously in combination studies using radiolabeled epratuzumab with unconjugated veltuzumab (16, 17), benefit can be derived from both agents given at their maximum effective and safe dosages. *In vitro* studies showed veltuzumab, even in the absence of cross-linking that is used to enhance signaling, accelerated apoptotic events initiated with Emab–SN-38. Thus, as long as the Emab–SN-38 conjugate was as effective as the anti-CD20 conjugate, selecting the Emab–SN-38 conjugate is a logical choice because it allows for a more effective combination therapy, even in tumors where one or both of the antigens are low in expression.

Because most ADCs using ultratoxic drugs are stably linked, we also tested a serum-stable, but intracellularly cleavable, anti-CD22–SN-38 conjugate, but determined it was 40- to 55-fold less potent than the CL2A linker. Others have examined a variety of ultratoxic drugs conjugated to anti-CD20 or anti-CD22 antibodies,

finding that internalizing conjugates are generally more active, but also observing that even slowly internalizing antibodies could be effective if the released drug penetrated the cell membrane (5, 46, 47). While the CL2A-type linker may be appropriate for SN-38, it may not be optimal for a more toxic agent, where even a small, sustained release in the serum would increase toxicity and compromise the therapeutic window.

Toxicology studies with the Emab-SN-38 in a relevant animal model are pending, but a previous examination of an anti-Trop-2-SN-38 conjugate in monkeys that express Trop-2 in a number of normal tissues found that it was safe at a cumulative human equivalent dose of approximately 40 mg/kg given in 1 week (11). Emab-SN-38 was active at a cumulative dose of 0.6 mg in mice bearing Ramos (75 µg twice weekly for 4 weeks), which extrapolates to a human dose of just 2.5 mg/kg. Thus, if Emab-SN-38 is tolerated at a similar level, it would have an ample therapeutic window to justify its evaluation in patients. Furthermore, an effective and safe dose of the anti-Trop-2-SN-38 conjugate was combined with a maximum tolerated dose of a ⁹⁰Y-labeled antibody without an appreciable increase in toxicity but with improved efficacy (48). Thus, the safety and efficacy profile of these SN-38 antibody conjugates are very favorable for other combination therapies.

Even though irinotecan is not used routinely for the treatment of hematopoietic cancers, SN-38 was as potent in lymphoma and leukemia cell lines as in solid tumors (11). In the WSU-FSCCL cell line, the specific and irrelevant IgG conjugates were significantly better than irinotecan, whereas in Ramos, the median TTP with the irrelevant conjugate was longer but not significantly better than irinotecan. These results are consistent with other studies that have shown that a nonspecific IgG is an excellent carrier for drugs and more potent *in vivo* than free drug or conjugates prepared with albumin or polyethylene glycol (PEG)-Fc (49). While the PEG-SN-38 conjugate had significant antitumor effects, it was given at its maximum tolerated amounts, ranging from 10 to 30 mg/kg SN-38 equivalents (37). In contrast, the maximum cumulative dose of SN-38 given over 4 weeks to animals bearing Ramos was only 1.6 mg/kg (i.e., dosing of 0.25 mg of Emab-SN-38 given twice weekly over 4 weeks) and this was nontoxic.

The specific therapeutic activity of Emab-SN-38 appeared to improve in cell lines with higher CD22 expression. For example, in Ramos, specific therapeutic

effects of Emab-SN-38 alone were recorded at 2 of the 3 different dose levels examined, and a sizeable number of tumors were completely ablated. In contrast, in WSU-FSCCL that had about 2.5-fold lower expression of CD22, Emab-SN-38 improved survival significantly compared with the irrelevant anti-CEACAM5-SN-38 conjugate in 1 of 2 studies. However, it is important to emphasize that when used in combination with unconjugated anti-CD20 therapy, Emab-SN-38 amplifies the therapeutic response. Thus, the combination of these two treatments could augment the response even in situations where CD22 is not highly expressed.

In conclusion, using the less-stable CL2A-SN-28 linker, Emab anti-CD22-SN-38 conjugate was equally active at nontoxic doses *in vivo* as a similar anti-CD20-SN-38 conjugate, despite the fact that CD20 expression was more than a log-fold higher than CD22. Therapeutic responses benefited by the combination of Emab-SN-38 with unconjugated Vmab anti-CD20 therapy, even when CD22 expression was low, suggesting that the combination therapy could improve responses in a number of B-cell malignancies when both antigens are present. The current studies suggest that this combination is very potent in diverse lymphoma and leukemia preclinical models, yet appears to have less host toxicity. Therefore, clinical studies with Emab-SN-38 are warranted.

Disclosure of Potential Conflicts of Interest

S.V. Govindan and T.M. Cardillo are employees of Immunomedics, Inc. D.M. Goldenberg has a financial interest in Immunomedics, Inc. No potential conflicts of interest were disclosed by the other authors.

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